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SUB-CELLULAR LOCALIZATION OF ANTIGEN E/2 OF *'ARACOCCIDIOIDES BRASILIENSIS*. AN IMMUNOENZYMATIC ELECTRON MICROSCOPY STUDY

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The species- or genus-specific soluble antigen E/2 of *Paracoccidioides brasiliensis* was detected in yeast cells of the fungus by means of an immunoenzymatic technique. Peroxidase-labelled anti-E/2 immunoglobulins revealed the antigen as irregular vesicles or spots in the cytoplasm and as a fine coating on the plasma membrane of yeast cells of seven Venezuelan strains of *P. brasiliensis*. The antigen deposits seem to move to the periphery of the cells as the cultures age.

Paracoccidioides brasiliensis, causal agent of paracoccidioidiomycosis, is an antigenically complex microorganism which possess at least 25 precipitant immunogens [4]. Early reports [5] have demonstrated the presence of species- or genus-specific antigens among such immunogens.

Two of the specific antigens have been isolated and monovalent (monospecific) antisera have been produced against them [6, 7]. The first, called E/1, showing alkaline phosphatase activity in immunoelectrophoregrams, is revealed only by a minor percentage of patients suffering mycologically confirmed paracoccidioidomycosis [6]. The second, named E/2, which manifests a weakly positive charge during agarose-gel electrophoresis at pH 8.2, showed immunological identity with the antigen of the band E [7], and reacted with all the human sera that formed this diagnostic immunoelectrophoretic band. Both mycelial and yeast phases of the fungus synthesized the substance. Antigen E/2 is therefore an important factor in the modulation of the host's immune response in paracoccidioidomycosis, and the determination of its cellular localization must be of interest in order to obtain fundamental information about the synthesis and release of this substance.

The present studies concern the application of the immunoperoxidase technique to demonstrate the subcellular distribution of antigen E/2. An experimental monovalent (monospecific) antiserum anti-E/2 was conjugated with horseradish peroxidase and used to examine the yeast cells of the fungus by electron microscopy.

MATERIALS AND METHODS

Microorganisms

P. brasiliensis strains No. 1313, 2111, 4678, 5858, 5931, 7273 and 9570 from the Centro de Micología Médica de Caracas, Venezuela, were examined. All these

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strains have been isolated from muco-cutaneous lesions of autochtonous patients and maintained on Sabouraud dextrose broth (Difco) at 4 °C for periods from 2 months to 3 years.

Yeast phase was obtained on Sabouraud dextrose agar supplemented with 20 % horse serum by incubation at 37 °C. The yeast cells were harvested after 3, 5, 7 and 10 days of incubation, fixed with 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer pH 7·4 for 2 h, washed three times in phosphate-buffered saline (PBS) and collected in a pellet by centrifugation for 15 min at 1200 r.p.m.

The yeast cells were treated according to the procedure used for *Candida albicans* by Borgers & De Nollin [1]. The pellets of *P. brasiliensis* were resuspended in a few drops of rat serum, followed by centrifugation; the supernatant was discarded and buffered glutaraldehyde was added. The pellets were frozen and fixed inmediately with a drop of buffered glutaraldehyde placed on the microtome stand; sections 7·5 μm thick were prepared and treated by immunoenzymatic procedures as described below.

Serological reagents

Antiserum to antigen E/2 was prepared in rabbits according to the method described by Yarzábal *et al.* [6]. Monospecificity of the serum was tested by gel immunodiffusion and bidimensional immunoelectrophoresis using crude soluble extracts of yeast and mycelial phases of *P. brasiliensis* as antigens.

Normal rabbit serum was obtained from a healthy male non-immunized rabbit. The immunoglobulins of rabbit anti-E/2 monovalent antiserum and normal rabbit serum were conjugated to horseradish peroxidase - HRPO - (type V1, Sigma Chemical Co.) by the method of Knowles *et al.* [3]. Briefly, 0·1 ml of 1% 1-fluoro-2,4-dinitrobenzene in absolute ethanol was added to 5 mg HRPO dissolved in 1·0 ml freshly prepared 0·3 M $NaHCO_3$, pH 8·1 and stirred gently for 60 min at room temperature. Subsequently, 1·0 ml of 0·08 M $NaIO_4$ was added and stirred for 60 min at room temperature. This solution was dialyzed against 0·01 M Na_2CO_3 buffer, pH 9·5, at 4 °C. Ten mg of the ammonium sulphate-precipitated immunoglobulins dissolved in 0·01 M Na_2CO_3 buffer, pH 9·5, was added and stirred gently at room temperature for 3 h. Five mg of $NaBH_4$ was added and the solution was allowed to stand overnight at 4 °C. The peroxidase-labelled immunoglobulin was separated from the unconjugated peroxidase by precipitation with an equal volume of cold saturated ammonium sulphate. The mixture was centrifuged at 600 × g for 30 min at 4 °C. The precipitated peroxidase-labelled immunoglobulins were dissolved in phosphate-buffered saline (PBS) and dialyzed against distilled water for 60 min and then against several changes of PBS at 4 °C. The final concentration was adjusted with PBS to between 5–7 mg ml^{-1} and stored at −20 °C.

Peroxidase labelling of P. brasiliensis *yeast cells*

The technique used was originally described by Graham & Karnovsky [2]. We used 5 mg of 3,3-diaminobenzidine (D.A.B.) in 10 ml Tris-HCl buffer, pH 7·6 and 1 drop of H_2O_2 for 10 min at room temperature; the D.A.B. oxidises to form an osmiophilic polymer. The sections of yeast cells were covered with either the peroxidase-labelled anti-E/2 immunoglobulins or the normal rabbit serum immunoglobulins for 3 h at room in a humid container. The sections were washed in distilled water, mounted in glycerol-jelly and observed directly with a light microscope. In all strains studied, sections 50 μm thick were post-fixed in 1% osmium tetroxide



dissolved in distilled water, dehydrated in alcohols and embedded in Epon. Grids were observed, with and without staining, in a Phillips 300 electron microscope.

All preparations were tested for endogenous peroxidase activity. Controls used normal rabbit labelled immunoglobulins instead of anti-E/2 specific immunoglobulins, and PBS instead of specific labelled immunoglobulins.

Results

In the first stage of growth of *P. brasiliensis* the intracytoplasmic dots and granules appeared irregularly distributed in the central vacuole and vesicles in its interior (Fig. 1). In the second stage the reaction product had a tendency to migrate to the lomasomes and the plasma membrane of the fungi (Figs 2 and 3). Finally the vesicles enlarged and came closer to the plasma membrance where the antigen-antibody reaction seemed to take place (Fig. 4).

Discussion

The incubation of yeast cells of 7 strains of *P. brasiliensis* with peroxidase-labelled immunoglobulins permitted detection of deposits of E/2 antigen at subcellular levels.

Labelled antibodies were observed in cells of all strains, forming irregular vesicles or spots in the cytoplasm and attached to the plasma membrane. The central intracytoplasmic spots seemed to move to the periphery of the cell and approached the internal face of the plasma membrane at the end of the period of observation (day 10).

Apparently the formation of the E/2 antigen begins at the level of the basal region of the cell, where the antigen is synthesized by ribosomes. It then penetrates in the vacuolar system in granular form or as a simple protein solution, becomes concentrated, and is surrounded by membranes for transport toward the apical part of the cell through transitory communications within the membranes. That is to say, the system of membranes distributed throughout the cellular cytoplasm would function as the equivalents of the endoplasmic reticulum and Golgi apparatus of *P. barsiliensis;* these organelles have never been unequivocally demonstrated in this type of micro-organism.

It should be emphasized that we never observed reaction product in the extracellular region which could represent non-specific uptake of the label by killed cells. We believe that the synthesis and excretion of the E/2 antigen is a dynamic process which becomes established from the earliest hours of the development of the fungus, since previous observations have demonstrated the presence of the antigen in cultures at very early stages of their development (Yarzábal, unpublished data).

In its migration toward the apex of the cell (Figs. 2 and 3), we observed the reaction product within vesicles, which approach the periphery before excretion at the level of the plasma membrane, apparently by a process of simple exocytosis. Alternatively, the E/2 antigen may remain for some time at the level of the plasma membrane and later pass to the exterior by means of the lomasomes of the fungus. These may penetrate deeply into the cytoplasm of the micro-organism, engulf the granules or aggregates of the protein and transport them to the exterior.

The time sequence of the synthesis and transport of the E/2 antigen and its intracellular movement would be very difficult to establish unless procedures of cell synchronization, fractionation or autoradiography were employed. Nor can we



Figure 1. 3 day culture of *P. brasiliensis*. The conjugate-D.A.B. precipitate is observed in a granular form and in the periphery of vesicles inside the central vacuole of the fungi (V). M = mitochondriae. × 3840.

Figure 2. 5 day culture of *P. brasiliensis*. The conjugate-D.A.B. precipitate is observed at the plasma membrane (PM). The precipitate is visible at the lomasomes (L), lower insert. 3500 × 2800.

Figure 3. 7 day culture of *P. brasiliensis*. The precipitate is observed at the periphery of the fungi (PM). × 3840.

Figure 4. 10 day culture of *P. brasiliensis*. The conjugate-D.A.B. precipitate is observed at the plasma membrane. The vesicles (V) have enlarged and are close to the plasma membrane. There is lack of continuity at this level (arrow) and the product of reaction can be seen outside the outer wall of the fungi (double arrow). M = mitochondriae. × 2800.



establish if the antigen is produced by all of the cells simultaneously or if its excretion involves a continuous, non-synchronized process.

Resumen

Mediante el uso de procedimientos inmunoenzimáticos y de la microscopía electrónica, se ha demostrado la distribución subcelular del antígeno E_2 en la fase levaduriforme de *Paracoccidioides brasiliensis*.

El antígeno fue observado a nivel de vacuolas y vesículas intra citoplasmáticas, así como en los lomasomas y en la membrana plasmática del hongo.

Ante estos hallazgos se postula que el antígeno E_2 podría ser sintetizado en organelas centrales (¿ ribosomas?), desplazándose luego hacia la periferia, para ser excretado a través de la membrana plasmática, por simple exocitosis o previa captación por los lomasomas.

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